# $\alpha_2$ High molecular mass cysteine proteinase inhibitor: HMr$\alpha_2$-CPI

## An inhibitor of human liver cathepsin H as probed by kinetic study

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HMr$\alpha_2$CPI was found to be an inhibitor of human liver cathepsin H by the measurement of the dissociation constant ($K_i$), the association rate constant ($k_1$) and the dissociation rate constant ($k_{-1}$) between the enzyme and the inhibitor. These data suggest that this protein-proteinase inhibitor can play a physiological role in the regulation of free cathepsin H.

*$\alpha_2$-Cysteine proteinase inhibitor* *Cathepsin H* *Kinetic study* *Physiological role*

## 1. INTRODUCTION

The inhibitory properties of HMr$\alpha_2$CPI are under investigation in our laboratory and it has been found that human liver cathepsin L is controlled by this protein-proteinase inhibitor [1]. A quantitative analysis of the interaction has been described [2]. On the other hand, this protein is also able to inhibit human liver cathepsin H (EC 3.4.22.16). The physiological significance of this phenomenon has been shown by a study of the equilibrium between the enzyme and the inhibitor. In spite of the lack of inhibition of human liver cathepsin B [3–5] HMr$\alpha_2$CPI seems to be a component in the regulatory pathway for lysosomal cysteine proteinases.

*Abbreviations:* HMr$\alpha_2$CPI, $\alpha_2$ cysteine proteinase inhibitor, high-$M_r$; C.H, cathepsin H (EC 3.4.22.16); $B_z$ArgNNap, *N*-benzoyl-DL-arginine-2-naphthylamide hydrochloride; ArgNNap, L-arginine-2-naphthylamide hydrochloride; LeuNNap, L-leucine-2-naphthylamide hydrochloride; ArgNMec, L-arginine-4-methyl-7-coumarylamide; E 64, L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane; p*I*, isoelectric point; DTT, 1,4-dithioerythreitol

## 2. MATERIALS AND METHODS

Pleural fluids were used for the preparation of HMr$\alpha_2$CPI. Disease-free human liver, stored at −20°C until required, was used for the preparation of cathepsin H. Papain, ficin, BzArgNNap, LeuNNap, ammonium sulphamate, sodium nitrite, *N*-(1-naphthyl)ethylene diamine and $\beta$-naphthylamine were purchased from Sigma (St Louis, MO). CNBr-activated Sepharose, Blue Sepharose (F3GA) Sephadex-G75 superfine, DEAE–Sephadex-A50 and Sephacryl S200 superfine were supplied by Pharmacia (Uppsala).

Aldolase activity was assayed with the Boehringer UV system (Mannheim). E 64 and ArgNMec were supplied by the Protein Research Foundation (Osaka).

### 2.1. *Purification and titration of HMr$\alpha_2$CPI*

This protein was isolated as in [1,2]. The effective molarity of the purified inhibitor was determined by titration with papain after active site titration of this proteinase by E 64 [2].

### 2.2. *Preparation of human liver cathepsin H*

The enzyme was purified by a modification of



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the method described for cathepsin B and L [1]. Homogenisation, precipitation with ammonium sulphate and dialysis were unchanged. These were followed by ion-exchange chromatography on a DEAE–Sephadex column. The purification was completed by two consecutive gel filtrations on a Sephadex-G75 (superfine) column (2.5 × 75 cm).

The purified enzyme was tested against ArgNNap, LeuNNap, BzArgNNap, aldolase and azocasein as described for cathepsin B and L in [1]. The activity against ArgNMec was carried out as in [7]. The effective molarity of this enzyme was determined by using E 64 as in [8]. Acrylamide gel electrofocusing and acrylamide gel electrophoresis in the presence of sodium dodecyl sulphate were performed as in [9] and [10], respectively.

All proteinase and inhibitor concentrations given in this paper refer to 'active concentrations'.

### 2.3. *Titrations of human liver cathepsin H by HMr$\alpha_2$CPI*

Cathepsin H ($0.1 \times 10^{-6}$ M) was incubated for 10 min at 25°C with increasing amounts of HMr$\alpha_2$CPI ($0.34 \times 10^{-8}$ M–$0.66 \times 10^{-6}$ M) in 0.1 M sodium phosphate buffer (pH 6.8), 2 mM EDTA, 1 mM DTT (final vol. 100 $\mu$l) before adding 10 $\mu$l of $3 \times 10^{-3}$ M ArgNNap ($S_o = 30\ k_m$). After 10 min at 40°C, the reaction was stopped and samples were processed as in [1].

### 2.4. $K_i$ *determination*

We have used the Easson-Stedman plot [11]. The non-linearity of the titration curve was obtained by working with a more dilute enzyme. This was possible with ArgNMec as a substrate.

Cathepsin H ($0.2 \times 10^{-7}$ M) was incubated with HMr$\alpha_2$CPI ($0.68 \times 10^{-8}$ M–$1.7 \times 10^{-7}$ M) under the conditions described above. Ten $\mu$l of $1 \times 10^{-4}$ M ArgNMec ($S_o = 6.6\ K_m$) was then added. After 15 min at 40°C, the residual enzymatic activity was measured as in [8].

The same experiment was carried out with 3 different substrate concentrations ($S_o$ = 0.66, 3.3, 6.6 $K_m$).

### 2.5. $k_i$ *determination*

The association rate constant was determined under second order conditions [12]. Equimolar concentrations of enzyme and inhibitor ($1 \times 10^{-8}$ M) were mixed in 5 ml (final vol.) of activation buffer at 25°C; 200 $\mu$l of the mixture was removed at the times 0, 1, 3, 5, 8, 10 min and assayed with ArgNMec (10 $\mu$l, $S_o = 3.3\ K_m$) which stops the reaction and measures the residual enzymatic activity.

### 2.6. $k_{-1}$ *determination*

The dissociation equilibrium study was carried out by complex dilution [13]. The complex between the enzyme and the inhibitor was obtained by allowing to react equimolar concentrations of both partners ($0.2 \times 10^{-6}$ M) during 10 min at 40°C (final vol. 100 $\mu$l).

This was followed by adding 1.9 ml of activation buffer. The increase in enzymatic activity was measured by removing 200 $\mu$l of this mixture at the times 0, 10, 20, 30, 45 and 60 min. The portions removed were assayed with ArgNMec (10 $\mu$l, $S_o$ = 3.3 $K_m$) as described before.

For the estimation of the total enzymatic activity, a control was made up in the same way, but without inhibitor.

Calculations were carried out by using:

$$\frac{\text{control} - \text{measurement}}{\text{control}} = \frac{1-a}{1} = \frac{EI}{EI_o}$$

## 3. RESULTS

### 3.1. *Titration of HMr$\alpha_2$CPI*

This purified inhibitor was found to be $6.9 \times 10^{-6}$ M active for a protein concentration of $16 \times 10^{-6}$ M; i.e., 43% of this inhibitor was active.

### 3.2. *Characterization of human liver cathepsin H*

The purification method gave an enzyme, purified about 600-fold. By acrylamide gel electrofocusing (pH gradient 3.5–10) one band at p*I* 6.20 was found when the staining was carried out with Coomassie brilliant blue. By an activity-staining method using ArgNNap the same pattern was obtained (fig.1). The most important properties of this enzyme are summarized in table 2: the high aminopeptidase and endopeptidase activities accompanied by a low proteolytic activity were in agreement with [6].

By active site titration, this enzyme was found to be 50% active. The operational molarity was $9.5 \times 10^{-6}$ M for a protein concentration of $19 \times 10^{-6}$ M. The specific activities reported in table 1 refer to the active fraction.

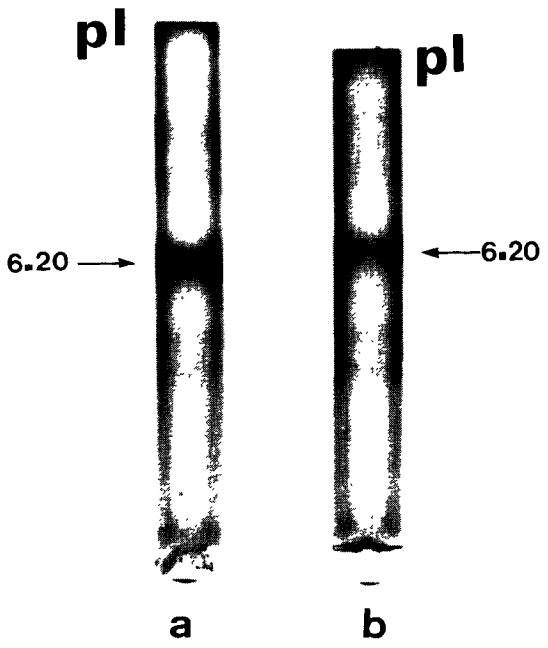


Fig.1. Gel electrofocusing of purified cathepsin H (25 $\mu$g sample). The experiment was carried out over a pH gradient 3.5–10.0. (a) Staining for enzymic activity with ArgNNap as a substrate. (b) Staining with Coomassie brilliant blue after incubation for activity staining.

### 3.3. *Titration of human liver cathepsin H by HMr$\alpha_2$CPI*

The inhibition of cathepsin H activity ($0.1 \times 10^{-6}$ M) upon addition of increasing amounts of HMr$\alpha_2$CPI gives a linear titration curve (fig.2). This result is consistent with a 1 : 1 association between both partners. As has been shown in [14] for reversible reactions, conditions for obtaining a linear titration curve are fulfilled when $E_o/K_i > 100$. These data allow us to suppose a low $K_i$ value.

Table 1

Some properties of human liver cathepsin H

| | | |
|---|---|---|
| Specific activities (units/mg at pH 6.8) against | | |
| ArgNMec | | 2.2 |
| ArgNNap | | 1.8 |
| LeuNNap | | 1.1 |
| BzArgNNap | | 1.0 |
| Azocasein | | 0.1 |
| Aldolase | | $0.14 \times 10^{-3}$ |
| $E^{1\%}_{280nm,1cm}$ | | 11.80 |
| $M_r$ value | | 26000 |
| Isozymes | Number | 1 |
| | p*I* | 6.20 |

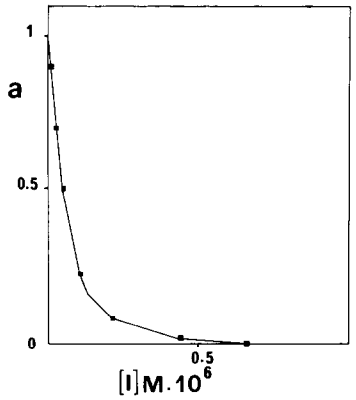


Fig.2. Residual fractional activity ($a = V_i/V_o$) of cathepsin H ($0.1 \times 10^{-6}$ *M*) on ArgNNap upon addition of HMr$\alpha_2$CPI ($0.34 \times 10^{-8}$ M–$0.66 \times 10^{-6}$ M).

### 3.4. K$_i$ *determination for cathepsin H and HMr$\alpha_2$CPI*

The dissociation constant ($K_i$) is determined by using the reversibility of the reaction. When working at a low enough enzyme concentration a reversibility of the proteinase-inhibitor association is observed [13]. Data were plotted as in [14] and the slope of the straight line obtained corresponded to app. $K_i$ (fig.3). In this case, substrate-induced dissociation was found by using 3 different substrate concentrations. When plotting app. $K_i$ *vs* $S_o$, the intercept on the ordinate gives the true $K_i$ value [14] (fig.3).

The interaction between human liver cathepsin H and HMr$\alpha_2$CPI was characterized by $K_i = 9.0 \times 10^{-10}$ M. On the other hand, the replot app. $K_i$ *vs* $S_o$ indicates a competitive inhibition [14].

### 3.5. k$_I$ *determination for cathepsin H and HMr$\alpha_2$CPI*

For the calculations of the association rate constant, concentrations of enzyme and inhibitor used are low enough to study the time-dependency of the reaction. Working under second-order conditions, and assuming a simple reversible

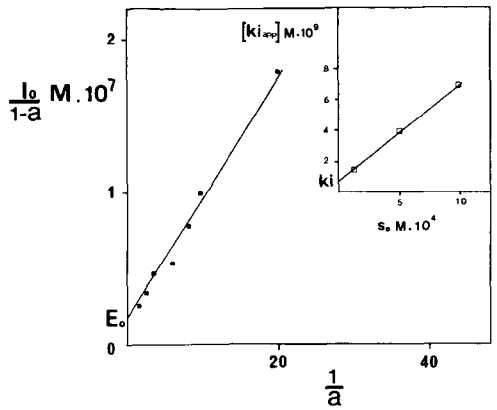


Fig.3. Determination of app. $K_i$ according to the following equation:
$\frac{I_o}{1-a} = \frac{K_i \text{ app}}{a} + E_o$ [11,14].
The residual fractional activity $a = V_i/V_o$ of cathepsin H ($0.2 \times 10^{-7}$ M) on ArgNMec was determined with increasing concentrations of HMr$\alpha_2$CPI ($0.68 \times 10^{-8}$–$1.7 \times 10^{-7}$ M). The insert shows the true $K_i$ value which was calculated from:

$$\text{app. } K_i = K_i + \frac{K_i S_o}{K_m} \text{ [14].}$$

equilibrium, the findings may be analyzed as in [13] (fig.4). We have found a $k_1$ value of $2.0 \times 10^{-6}$ $M^{-1} \cdot s^{-1}$). We have also estimated the delay time, i.e., the time required for complete inhibition of a proteinase in vivo [13], by using an ex-

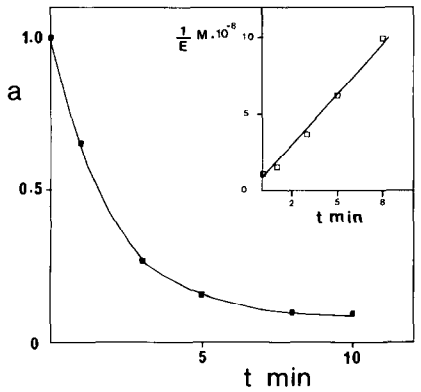


Fig.4. Time-dependency of the inhibition of human liver cathepsin H by HMr$\alpha_2$CPI ($1.0 \times 10^{-8}$ M for both partners). The insert is a second order replot according to:

$$\frac{1}{E} = \frac{1}{E_o} + k_1 t \text{ [13]}$$

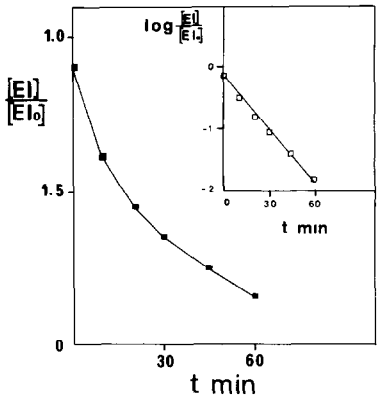


Fig.5. Dissociation of the cathepsin H–HMr$\alpha_2$CPI complex by dilution. The enzyme and the inhibitor ($0.20 \times 10^{-6}$ M) were preincubated during 10 min at 40°C (100 $\mu$l final vol.); 1.9 ml of activation buffer was then added and enzymatic activity was monitored against time. The insert is the first order replot according to:

$$\log \frac{EI}{EI_o} = -k_1 t \text{ [13]}$$

travascular concentration of $5.5 \times 10^{-6}$ M for HMr$\alpha_2$CPI [2]. This result was found to be 2 s.

### 3.6. $k_{-1}$ *determination for cathepsin H and HMr$\alpha_2$CPI*

For the measurement of the first order dissociation rate constant, the enzyme and the inhibitor were mixed at equivalent concentrations ($0.2 \times 10^{-6}$ M). High concentrations were used in order to carry out a complete association between both partners. After a sufficient preincubation time, a large volume of buffer was added to induce the dissociation process. The increase in enzymatic activity is followed as a function of time. The results are plotted as in [13]. We have found a $k_{-1}$ value of $1.0 \times 10^{-3} \cdot s^{-1}$ (fig.5). The stability time, i.e., the time during which the complex remains undissociated in vivo [13], was found to be 3 min.

## 4. DISCUSSION

HMr$\alpha_2$CPI inhibits human liver cathepsin H. The results reported here are consistent with this interaction being physiologically significant. The inhibitor binds the enzyme fast enough and the $I_o$:$K_i$ ratio is very high (table 2). If this enzyme is liberated during inflammatory states, it will be

Table 2

Kinetic constants for the interaction of HMr$\alpha_2$CPI with cathepsin H

| $K_i$ (M) | $K_1$ ($M^{-1} \cdot s^{-1}$) | Delay time (s) | $K_{-1}$ ($s^{-1}$) | Stability time (min) | $I_o/K_i$ |
|---|---|---|---|---|---|
| $9.0 \times 10^{-10}$ | $2 \times 10^6$ | 2 | $1.0 \times 10^{-3}$ | 3 | $6.0 \times 10^3$ |

complexed by HMr$\alpha_2$CPI. It is surprising that the function of this inhibitor has not been discovered before. The lack of inhibition of human liver cathepsin B [3–5] may explain these negative investigations. We have found that human liver cathepsin L is also inhibited by HMr$\alpha_2$CPI with a high efficiency [2]. The specificity of this inhibitor would be restricted to both lysosomal cysteine proteinases?

On the other hand, the tight binding of papain by HMr$\alpha_2$CPI ($K_i \sim 10^{-12}$ M) demonstrates the existence of a structural analogy between plant and mammalian cysteine proteinases, cathepsin H and L seeming to have a common binding site for HMr$\alpha_2$CPI with papain. This site, however, does not seem to exist on cathepsin B. But cathepsin B and papain have an extremely high sequence analogy in the active site region [15]. Thus it seems that the binding site for HMr$\alpha_2$CPI is not located in the active site region.

We have found a competitive inhibition for both lysosomal cysteine proteinases ([2], here). This result can be explained by bound inhibitor overlapping the active site. This is consistent with the high-$M_r$ of HMr$\alpha_2$CPI. Cathepsin H is an endopeptidase as well as an aminopeptidase and both activities may provoke tissue damage during inflammatory reactions. The presence of HMr$\alpha_2$CPI outside the circulation (e.g., in pleural and ascitic fluids) dispels any doubt on the physiological role of this inhibitor. However, this protein is not an 'acute phase reactant' in the human [2], but in the rat it is identical with $\alpha_1$ acute phase globulin [16]. The recent report of [17] shows that the sequence of cathepsin H is more closely related to that of papain than the sequence of cathepsin B. Our findings fit in well with the structural homology between papain and cathepsin H and underline the structural differences between papain and cathepsin B.

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